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Towards *in vivo* mutation analysis: Knock-out of specific chlorophylls bound to the light-harvesting complexes of *Arabidopsis thaliana* — The case of CP24 (Lhcb6)☆

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ABSTRACT

In the last ten years, a large series of studies have targeted antenna complexes of plants (Lhc) with the aim of understanding the mechanisms of light harvesting and photoprotection. Combining spectroscopy, modeling and mutation analyses, the role of individual pigments in these processes has been highlighted *in vitro*. In plants, however, these proteins are associated with multiple complexes of the photosystems and function within this framework. In this work, we have envisaged a way to bridge the gap between *in vitro* and *in vivo* studies by knocking out *in vivo* pigments that have been proposed to play an important role in excitation energy transfer between the complexes or in photoprotection. We have complemented a CP24 knock-out mutant of *Arabidopsis thaliana* with the CP24 (*Lhcb6*) gene carrying a His-tag and with a mutated version lacking the ligand for chlorophyll 612, a specific pigment that *in vitro* experiments have indicated as the lowest energy site of the complex. Both complexes efficiently integrated into the thylakoid membrane and assembled into the PSII supercomplexes, indicating that the His-tag does not impair the organization *in vivo*. The presence of the His-tag allowed the purification of CP24-WT and of CP24-612 mutant in their native states. It is shown that CP24-WT coordinates 10 chlorophylls and 2 carotenoid molecules and has properties identical to those of the reconstituted complex, demonstrating that the complex self-assembled *in vitro* assumes the same folding as in the plant. The absence of the ligand for chlorophyll 612 leads to the loss of one Chl *a* and of lutein, again as *in vitro*, indicating the feasibility of the method. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

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1. Introduction

In the first step of photosynthesis, light energy is harvested by the antenna complexes associated with Photosystem I (PSI) and II (PSII). In plants and green algae these complexes belong to the Lhc (Light harvesting complex) multigenic family. Lhcs are composed of a relatively small apoprotein (20–30 kDa) which coordinates up to 15 kDa of pigments: chlorophylls *a* (Chl *a*) and *b* (Chl *b*), β-carotene, lutein, neoxanthin, and violaxanthin [1]. Twelve proteins compose the outer antenna system of PSI and PSII in *Arabidopsis thaliana* [2] and twenty are present in the green algae *Chlamydomonas reinhardtii* [3]. These complexes are believed to have a dual role: (i) light-harvesting in light-limiting conditions, where they absorb light and efficiently transfer excitation energy to the reaction center to promote charge separation [4], and (ii) photoprotection in high light conditions, when the absorbed light cannot be used for photochemistry and needs to be dissipated to avoid oxidative damage [5]. The properties of several members of the Lhc family have been studied in detail *in vitro*, using complexes purified from the membranes and/or with recombinant complexes reconstituted *in vitro* (see [6–8] and references therein). *In vitro* reconstitution [9] has also allowed the production of complexes with different pigment composition and mutants lacking specific pigments (for CP24 see [10–12]). The studies on isolated complexes have provided information about the properties of the individual pigments and their possible role in light-harvesting and photoprotection (*e.g.* [13–15]). However, both processes require the simultaneous presence of several photosynthetic complexes and a series of factors that cannot be reproduced *in vitro*. Therefore, it is necessary to verify *in vivo* the experimental data on isolated complexes, which until now has not been possible.

☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

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In addition, only a few Lhcs have been purified from thylakoids to homogeneity in what is believed to be their native state; the large majority of them have proven difficult to isolate with all cofactors. This is certainly the case for the minor antenna complexes of higher plants (CP29, CP26 and CP24), which are purified in several steps using high detergent concentrations [16–18] that can lead to loss of pigments. Indeed, it has been shown that LHCII trimer, the major antenna complex in plants and algae that is far more stable than the monomeric complexes, still loses one carotenoid and one Chl when purified in the same conditions used for the minor antenna complexes [19], thus pigments loss from more labile complexes is very likely. This hypothesis is supported by studies of the crystal structure of CP29 [20]: The biochemical and spectroscopic properties of the crystallized protein are different from that of the starting material and of all other CP29 preparations found in literature (*e.g.* [17,21–23]). In particular, the crystallized complex coordinates a higher number of pigments and shows a lower Chl *a*/*b* ratio [20]. These differences suggest that the fraction that crystallizes is the more intact subset of the ensemble and it retains all Chls associated with this protein in the native membrane. In the case of CP24, purification has proven to be even more difficult. It is reported that the CP24 complex purified from plants coordinates only a few Chls [24]. More information about the properties of CP24 has been obtained from the analysis of the complex reconstituted *in vitro*, which suggested that CP24 binds 10 Chls and 2 carotenoid molecules [10,11,25]. However, the principle that the reconstituted complexes are identical to the native ones is not fully accepted (several anonymous referees). In addition, it is known that the reconstituted LHCII coordinates only 3 carotenoids, even in its timeric state [26], and the peripheral violaxanthin site is empty [27,28]. In addition, the Chl *a*/*b* ratio of the recombinant complex strongly depends on the Chl *a*/*b* ratio of the reconstitution mix [29]. To get the most reliable information about the properties of the complexes in their native state, it is thus necessary to purify them to homogeneity from plants using a very mild purification procedure.

In this work, we have explored the possibility to overcome the limitations of the purification of individual antenna complexes. At the same time, we have produced *A. thaliana* mutants lacking specific pigments in a targeted binding site in order to test suggestions regarding their role in light-harvesting and photoprotection directly in plants. With these aims, we have transformed the CP24 knockout (CP24ko) mutant of *A. thaliana* with the *Lhcb6* gene fused with a His-tag in order to purify the complex using very mild detergent conditions. In addition, we have transformed the plants with a modified *Lhcb6* gene which lacks the ligand for Chl 612. According to *in vitro* experiments, this mutant lacks the lowest energy Chl and lutein in the L1 site [11], and so represents a good test to check the feasibility of mutation analysis *in vivo*. Finally, the refolding of the mutated protein in the plant with the native pigment complement allows evaluation of the reliability of the results obtained by *in vitro* reconstitution.

2. Materials & methods

2.1. Cloning of CP24 genomic sequence, Arabidopsis transformation and screening

The genomic DNA fragment consisting of 324 base pairs upstream of the transcriptional start site, plus the gene for CP24 up to the stop codon (1094 bp) was amplified by PCR from genomic DNA isolated from *A. thaliana* ecotype Columbia-0. Primers were designed to add a spacer of 2 Gly, a tail of 6 His residues, and a stop codon in frame at the C-terminal. The primers included *attB* sites allowing cloning by the Gateway cloning system (Invitrogen). Primer sequences were as follows: F-ggggacaagtttgtacaaaaaagcaggctATCATGTAATGTGTAATCTAAACGC, R-ggggaccactttgtacaagaaagctgggtTCAatggtgatggtgatggtgtccaccCAAACCAAGAGCACCGAGAGGC.

The amplification product was recombined into plant destination vector pEarlyGate302 [30] and the sequence was verified. The genomic sequence is high-lighted by capital letters. In the reverse primer the stop codon is high-lighted as well. attB sites, spacer and His-tail are indicated in small letters.

The Chl 612 binding site was mutated by site-directed mutagenesis (Strategene) by changing the polar residue histidine191 into a phenylalanine [11] and the sequence was verified.

Arabidopsis plants with a T-DNA insertion in the *lhcb6* gene (SALK_077953, Columbia-0 ecotype) were obtained from NASC collection and a homozygous line (CP24ko) was identified by Western blotting using CP24 antibody (Agrisera). CP24ko plants were grown in a growth chamber under long-day conditions for 8–10 weeks and transformed by the floral dip method [31,32].

10-Day seedlings were screened for resistance to applications of the herbicide Basta (250 mg/l). The expression of the CP24 transgene was assessed by Western blot. For each genotype 20 independent transformants (T1 generation) were self-fertilized, and five homozygous CP24-His lines (T2 generation) and four Chl612 mutant lines were identified by segregation of Basta resistance. The two lines are called CP24-WT-His and CP24-612-His in the following.

2.2. Thylakoid and grana membrane isolation

Thylakoids and grana membranes were prepared from WT Col-0, CP24ko, CP24-WT-His and CP24-612-His mutant plants. Plants were

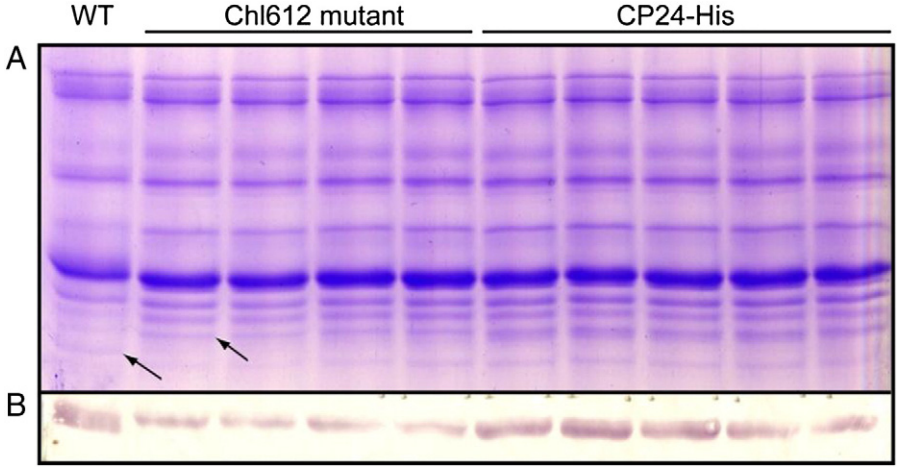

Fig. 1. Analysis of the thylakoid membranes from independent plant lines expressing CP24-His proteins. A) SDS-PAGE: the gel was loaded with 3 μg of Chls of each line and stained with Coomassie blue, the arrows indicate the position of CP24. B) Western blot with colorimetric detection of CP24. Five independent WT lines and four independent Chl612 mutant lines are shown.

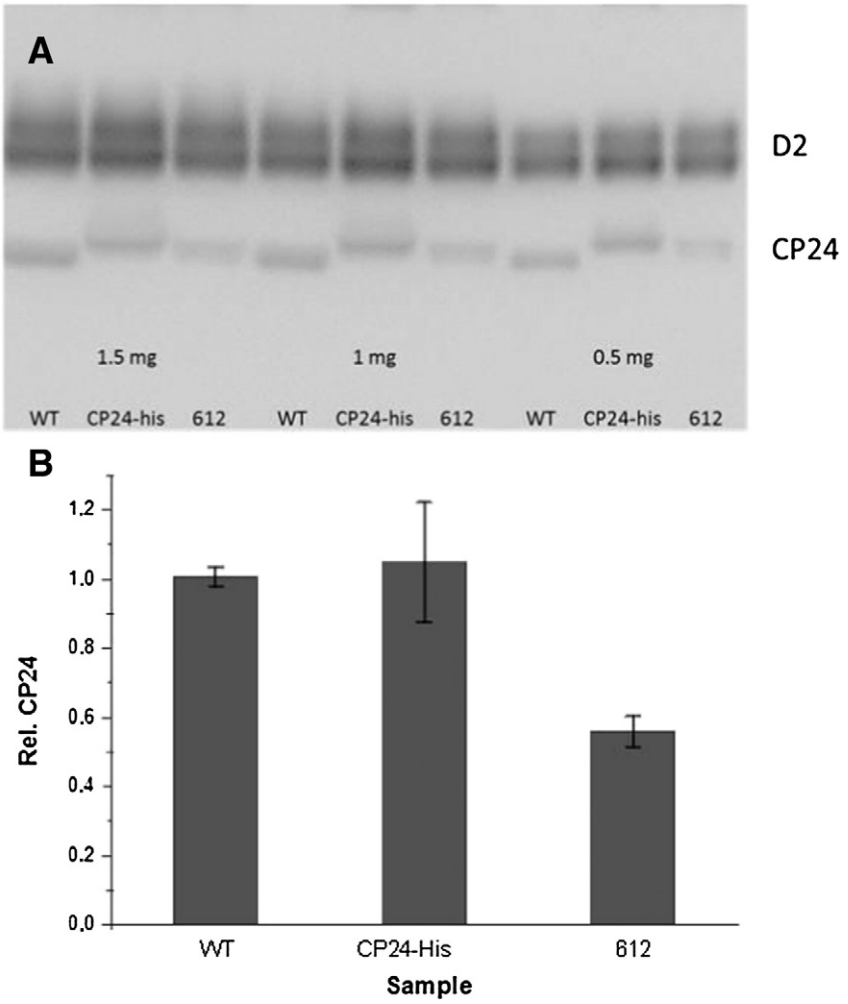

Fig. 2. CP24 relative amount in the His-tagged mutants. (A) Western blot of thylakoid membranes using antibodies against D2 and CP24. Plants of different genotypes were grown in the same light conditions. (B) The amount of CP24 was determined from densitometry on the 3 replicates and normalized to the D2 signal. The label "612" refers to the thylakoids from the "CP24-612" line.

grown under 130 μmol photons m^{-2} s^{-1} of light (8 h/day) at 21 °C. Grana membranes were prepared from isolated thylakoids by solubilization with Triton X100 according to [33] with few modifications as in [34].

2.3. CP24 isolation

Thylakoids membranes of CP24-WT-His and CP24-612-His were washed with 5 mM EDTA, resuspended in 10 mM HEPES pH 7.6 at a chlorophyll concentration of 1 mg/ml and then solubilized with 0.6% dodecyl-α-D-maltoside (α-DDM), 10 mM HEPES pH 7.6. Unsolubilized material was removed by centrifugation at 12,000 *g* for 10 min at 4 °C, and the supernatant was then fractionated by ultracentrifugation on a 0.1–0.9 M sucrose gradient containing 0.03% α-DDM and 10 mM HEPES pH 7.5 (19–21 h at 280,000 *g* at 4 °C with a SW41 rotor). The green band corresponding to the pool of monomeric antenna complexes was harvested with a syringe, diluted with an equal volume of dilution buffer (20 mM HEPES pH 7.6, 0.4 M NaCl, 0.03% α-DDM) and loaded on a Ni-Sepharose column for affinity purification of the His-tagged protein. The column was equilibrated and washed with a binding buffer (20 mM HEPES pH 7.6, 0.2 M NaCl, 0.03% a-DDM) until the absorbance was close to baseline. Monomeric CP24 was eluted by adding 0.25 M imidazole. CP24 was reconstituted as in Passarini et al. [11].

2.4. Steady-state spectroscopy

Absorption spectra were recorded on a Varian Cary 4000 UV–vis-spectrophotometer. For 77 K measurements a home-built liquid N_2 cooled low-temperature device was used. Fluorescence spectra were recorded at room temperature on a Fluorolog 3.22 spectrofluorimeter (Jobin Yvon-Spex). Circular-dichroism (CD) spectra were recorded at 20 °C on a Chiraschan-Plus spectropolarimeter (Applied Photophysics). All measurements were performed in 10 mM tricine pH 7.8, 0.03% α-DM and 0.5 M sucrose or with 67% (w/v) glycerol for 77 K measurements.

2.5. Pigment analysis

The pigment complement of the complexes was analyzed by fitting the acetone extract spectrum with the spectra of the individual pigments and by high pressure liquid chromatography [35]. The values of Chl *a*/*b* and Chl/car were obtained from the fitting of the acetone extract, while the relative values of the xanthophylls were obtained by HPLC analysis.

2.6. SDS page and immunoblotting

SDS-PAGE electrophoresis was performed as in [36], but using an acrylamide/bis-acrylamide ratio of 75:1 and an acrylamide + bis-

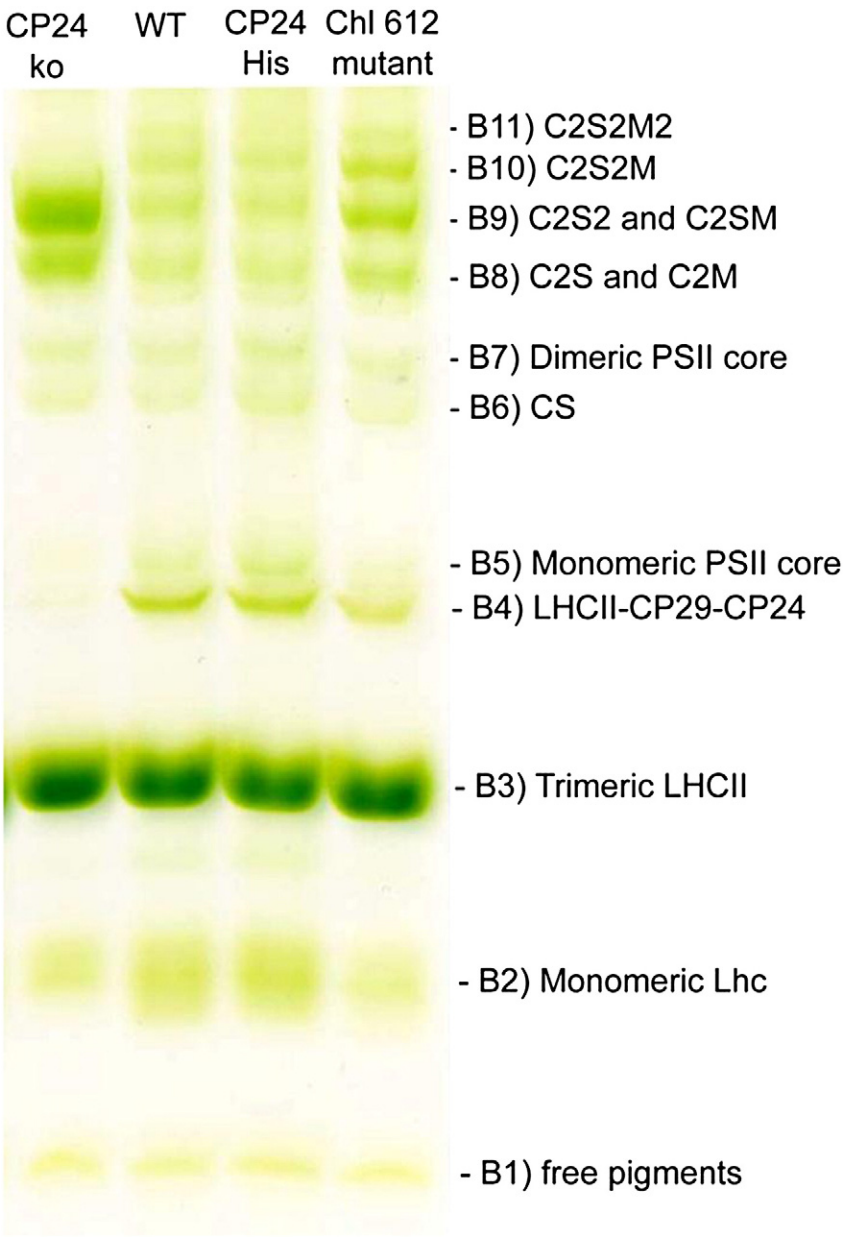

Fig. 3. PSII organization in wild-type plants and complemented lines. Native Deriphat-PAGE of grana membranes: the gel was loaded with equal amount of Chls in each line. The content of each band is indicated on the basis of earlier work [34]. CP24 is present in bands 2, 4 and 8–11.

acrylamide concentration of 15.5%. Urea 6 M was also incorporated into the gels. Proteins were detected after blotting using specified antibodies (www.agrisera.se) by colorimetric BCIP/NBT detection or Chemiluminescent detection.

2.7. Deriphat PAGE analysis

Nondenaturing Deriphat-PAGE was performed following the method described previously [37], but using 3.5% (w/v) acrylamide (38:1 acrylamide/bisacrylamide) in the stacking gel and resolving gel, and an acrylamide concentration gradient from 4.5 to 11.5% (w/v) stabilized by a glycerol gradient from 8 to 16%. Thylakoids concentrated at 1 mg/mL chlorophyll were solubilized with a final 0.8% α-DM, and 30 mg of chlorophyll were loaded in each lane.

3. Results and discussion

3.1. Engineering Arabidopsis for the purification of the native CP24 light-harvesting complex and in vivo mutation of Chl 612 binding site

The gene coding sequence for CP24 was amplified from genomic DNA extracted from *A. thaliana* (see Materials & methods for details). The primers were specifically designed to include the promoter sequence and a polyhistidine tag in frame to the last exon in lieu of the natural stop codon. The presence of the native promoter should permit physiological expression of the transgene while limiting a potential silencing response of the plant that can occur with strong constitutive promoters [38]. The amplified genomic fragment was cloned in a vector for Agrobacterium-mediated plant transformation conferring resistance to the herbicide Basta. CP24ko plants, identified by immunoblotting

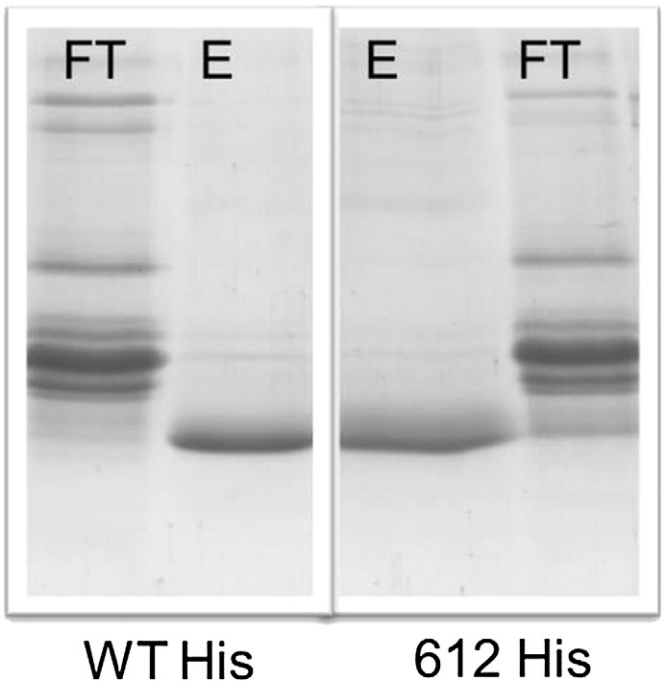

Fig. 4. Protein composition of samples purified by Ni-affinity chromatography. SDS-PAGE stained with Coomassie blue of the flow through (FL) and the eluted fractions (E) of CP24-WT-His and CP24-612-His.

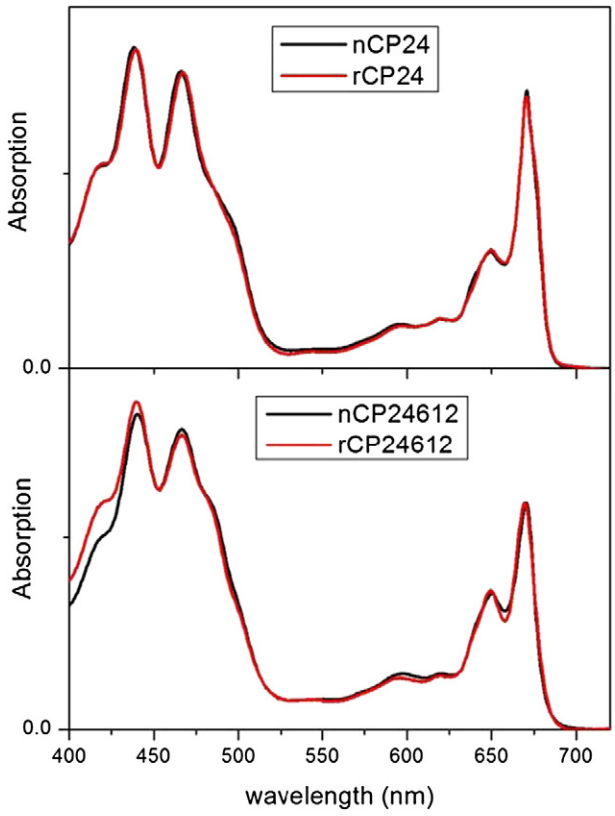

Fig. 5. Comparison of the absorption spectra of CP24 native (n) and recombinant (r) at 77 K (A) CP24-WT-His; (B) CP24-612-His.

from lines segregating a T-DNA insertion in the *lhcb6* gene, were then transformed using the floral dip method.

In order to investigate the properties of Chl 612 *in planta*, another set of CP24ko plants was transformed with the gene of CP24 lacking the ligand of Chl 612. Finally, five independent transgenic lines expressing the His-tagged CP24 (CP24-WT-His) and four expressing the protein mutated at site 612 (CP24- 612-His mutant, also carrying the His-tag) were selected by screening for the resistance to herbicide and the presence of the protein as assessed by immunoblotting.

3.2. The His-tagged CP24 complexes were expressed at physiological level and localized in the thylakoids

To determine if the proteins were correctly targeted to the thylakoid membranes, the thylakoid membranes of the transgenic lines were purified and their protein composition was analyzed by SDS-PAGE (Fig. 1A) and Western blotting (Fig. 1B). The His-tagged CP24 proteins recognized by the specific antibody were present in all lines, indicating that the proteins, which are expressed in the nucleus and synthesized in the cytoplasm, were correctly addressed to the thylakoid membranes.

Judging from the signal of the antibody, the relative amount of CP24-His proteins, on a chlorophyll basis, seemed substantially the same in all independent CP24-WT-His plants compared with the wild-type, suggesting that the content of CP24 is finely regulated in the thylakoids. The relative amount of CP24-His was more precisely evaluated using the D2 protein as internal standards by densitometry analysis of the chemiluminescent signal (Fig. 2). The different mobility of CP24-WT, CP24-His and CP24-612 in the gel is due to the presence of the His-tag. The results indicate the presence of an equal amount of CP24-His compared with the wild-type, while the level of CP24 in the CP24-612-His mutant was clearly reduced.

3.3. The presence of the His-tag does not influence the organization of the supercomplexes

It has been shown that in the CP24ko mutant the composition and organization of the PSII supercomplexes are different than in the WT plants. In particular, the largest particle observed contained only one LHCII trimer per core complex, in addition to CP29 and CP26 (C_2S_2 supercomplex), while a second trimer normally observed in *A. thaliana* membranes (trimer M according to the nomenclature of [39]), was not associated with it [40,41]. To check the composition of the PSII supercomplexes in the CP24-His plants, mildly solubilized grana membranes were analyzed by native Deriphat-PAGE (Fig. 3). Several bands corresponding to different PSII components (from B1 to B7) and to whole supercomplexes of increasing size (from B8 to B11) were resolved, in agreement with the fractionation in sucrose density gradient [34]. In contrast with CP24ko, both His-tagged mutants clearly

Table 1
Pigment composition of CP24 complexes as determined by HPLC and by fitting of the acetone extracts (see Materials & methods for detail). Data are normalized to the suggested number of Chls reported in the last column. Data are means of at least 3 replicas.

Sample	Chl *a*/*b*	Chls/Cars	Vio	Lut	Chl *b*	Chl *a*	Total chls
CP24-WT-His	1.03 ± 0.013	5.34 ± 0.03	1.02 ± 0.01	0.87 ± 0.02	4.99 ± 0.04	5.01 ± 0.04	10
CP24-612-His	0.80 ± 0.002	6.94 ± 0.03	1.12 ± 0.01	0.20 ± 0.03	4.96 ± 0.06	4.04 ± 0.06	9

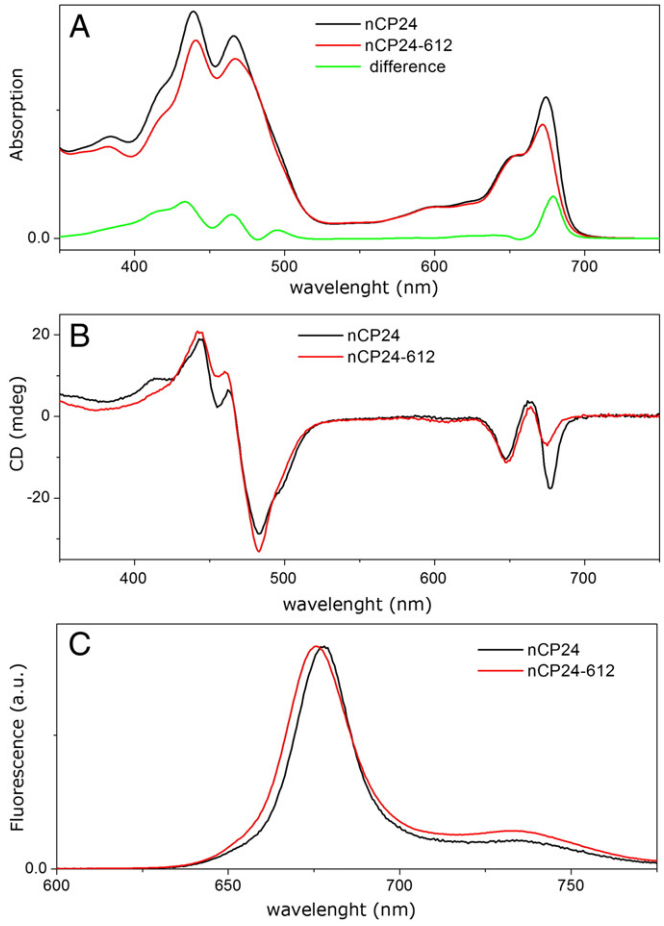

Fig. 6. Spectroscopic properties of CP24-WT-His and CP24-612-His. (A) RT absorption spectra normalized to the number of Chls in each complex. The difference spectrum is also shown; (B) CD spectra of the same complexes as in panel A normalized to the absorption; (C) Fluorescence emission spectra of the same complexes as in panel A with excitation at 500 nm normalized to the maximum.

showed a wild-type-like organization/composition of the PSII supercomplexes. In particular, they differ from the CP24ko for: 1) the presence of B4 band, which is a stable complex composed of CP24, CP29 and LHCII M trimer [42]; and 2) the presence of PSII supercomplexes, B10 and B11, containing one and two copies of LHCII M trimer respectively [34]. The lower amount of B4 band in the CP24-612-His mutant can be fully attributed to the lower expression level of CP24 in these plants. On the basis of these results, we can conclude that CP24-His is correctly assembled in the PSII supercomplex, and thus that the presence of the His-tag does not interfere with the protein–protein interactions.

3.4. Purification and characterization of CP24 (WT-his and 612-His) complexes

With the aim of purifying CP24, thylakoid membranes isolated from CP24-His plants were mildly solubilized and fractionated on sucrose density gradients. The band corresponding to the pool of monomeric antenna complexes was then subjected to chromatography on a Nickel column (see Materials & methods for details). The procedure allowed us to obtain pure CP24, as shown in Fig. 4, in a suitable amount for complete biochemical/spectroscopic characterization. The purity of CP24 (Fig. 4) was >95% as assessed by densitometry.

To determine the pigment content of the complexes, Chls *a*/*b* and Chls/Cars ratios were measured by fitting the spectrum of the acetone extract of the samples with the spectra of individual pigment species in the same solvent. In addition, the relative amount of each xanthophyll was measured by HPLC. The pigment composition is reported in Table 1. Considering that CP24 lacks Chl 613 and 614 binding sites [11], and that the monomeric complexes do not seem to contain Chl 601 and 605 [20], it seems reasonable that CP24 coordinates 10 Chls (5 Chls *a* and 5 Chls *b*) and 2 xanthophylls (1 lutein and 1 violaxanthin). However, based on the Chl/car stoichiometry it is not possible to exclude the presence of one additional Chl, which would then be a mixed binding site with identical affinity for Chl *a* and *b*. The Chl *a*/*b* ratio of the CP24-612 mutant was lower than that of the WT, corresponding to the loss of at least 1 Chl *a*, indicating that a Chl *a* is coordinated in site 612. This conclusion is based on the assumption that the mutated complex does not lose Chl *b* as supported by the identical shape of the absorption spectrum of CP24-WT-His and CP24-612-His in the Chl *b* absorption region. The CP24-612 complex also shows loss of lutein, most likely from the L1 site which is located in close proximity of Chl 612, while the amount of violaxanthin remains at the levels of the WT, suggesting that violaxanthin is located in the L2 binding site. Notably, the pigment composition of both complexes is identical to that of the same complexes reconstituted *in vitro* [11] using in the reconstitution mixture a Chl *a*/*b* ratio of 3 and all carotenoids present in the membrane. This also indicates that the complex self-assembled *in vitro* maintains the same affinity of the binding sites for the different pigments as the native system.

Absorption spectra at 77 K of CP24-WT-His and CP24-612-His purified from plants are presented in Fig. 5, together with the spectra of the recombinant complexes reconstituted *in vitro* [11]. The spectra of the native and reconstituted complexes are virtually identical, indicating a conserved pigment organization. In Fig. 6 the absorption at RT, circular dichroism and fluorescence spectra of CP24-WT-His and CP24-612-His are presented. It is shown that the CP24-612-His loses absorption at 679 nm, as was the case for the reconstituted complex [11]. The blue shift of the fluorescence of the mutant with respect to that of the WT and the loss of the red most signal in CD also confirm that Chl 612 is strongly interacting with another Chl and it is responsible for the lowest energy state also *in vivo*, fully confirming the results obtained *in vitro* [11,26].

4. Conclusions

In this work, we have created *A. thaliana* plant lines expressing CP24 protein fused with a C-terminal His-tag in a CP24ko background. We show that the recombinant protein is correctly localized to the thylakoid membrane and importantly, the wild-type pattern of photosynthetic complex and supercomplex formation that is disrupted in CP24ko plants is restored. In addition, we have introduced a mutated version of the gene which lacks the ligand for Chl 612 into CP24ko plants, thus establishing for the first time an *in vivo* method to explore the function of key pigments in the processes of excitation energy transfer and heat dissipation.

The utilization of the His-tag for purification of the CP24 complexes allowed for milder conditions than previously required for preparation of native complexes, mitigating potential pigment loss. Notably, the solubilization conditions used here for the purification of CP24 do not lead to pigment loss in LHCII (Xu et al. in preparation). This allows us to interpret our results as indicating that CP24 binds 10 Chls, one violaxanthin and one lutein, in sites L2 and L1, while both sites N1 and V1, which in LHCII are occupied by neoxanthin and violaxanthin/lutein [43], are not present in CP24. This is in agreement with the fact that CP24 lacks the Tyr residue which stabilizes the neoxanthin binding [44] and that the V1 site is not observed in monomers [20].

The data also show that the properties of the complexes purified from plants are virtually identical to those obtained by *in vitro* reconstitution, when the reconstitution is done using pigment mixtures with a Chl *a*/*b* ratio of 2.9–3.1, the value observed in the thylakoid membrane of *A. thaliana* grown in normal light conditions [45]. It is also shown that the Chl organization upon *in vitro* and *in vivo* folding is identical for both WT and mutant complexes, and that Chl 612 is the lowest

energy state of the complex *in vivo* as well as *in vitro*. In addition, we confirm that L1 site is occupied by lutein and L2 site is occupied by violaxanthin, again in agreement with the results *in vitro* [11], indicating that the affinity of the binding site is preserved in the *in vitro* reconstitution.

Finally, the possibility to knock out individual pigments *in vivo* allows information to be obtained at the molecular level while still analyzing the system at high level of complexity. This is important because most photosynthetic processes require the presence of many players and thus the exact mechanisms need to be verified directly in the plant, but this is normally too complex to allow for molecular details. Mutation analysis *in vivo* bridges this gap.

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